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"*Candidatus* Midichloria" Endosymbionts Bloom after the Blood Meal of the Host, the Hard Tick *Ixodes ricinus*▽

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"*Candidatus* Midichloria mitochondrii," an intracellular symbiont of the tick *Ixodes ricinus*, is the only described organism able to invade the mitochondria of any multicellular organism. We used quantitative PCR to examine cycles of bacterial growth and death throughout the host's development and found that they correspond with the phases of engorgement and molt, respectively.

The European hard tick *Ixodes ricinus* is a vector of numerous pathogenic microorganisms (11). While these pathogens have been extensively studied, less attention has been given to the tick's symbionts, which may have an important effect on host biology. "*Candidatus* Midichloria mitochondrii" is an intracellular alphaproteobacterial symbiont that inhabits the germ line cells of its female host (3, 5, 13, 16). It has the unique ability to invade and destroy mitochondria within ovarian cells (12). "*Ca.* Midichloria mitochondrii" has been detected in 100% of the examined *I. ricinus* females across its geographical distribution and in 44% of males (9). The basis of the symbiosis is not well understood. *I. ricinus* tick lines raised in the laboratory apparently lose the symbiont but continue to survive and reproduce (9). Thus, the symbiosis does not appear to be obligatory. We used real-time quantitative PCR (qPCR) to examine the population dynamics of the symbiont during the host's life cycle, which involves a blood meal at each of the larval, nymph, and adult stages. qPCR has successfully been used to obtain insights into the basis of a number of other intracellular symbiont-host interactions (1, 10, 15).

Adult ticks were collected in the woods surrounding Ceske Budejovice (Czech Republic). All males and some of the females were preserved in 100% ethanol, while the other females were fed on guinea pigs. Starting with these females, an entire life cycle was completed. DNA was extracted from ethanol-preserved ticks as described previously (3). Sybr green real-time qPCR protocols were designed for the following: (i) the *gyrB* gene of "*Ca.* Midichloria mitochondrii" (primers CTTG AGAGCAGAACCACCTA [forward] and CAAGCTCTGCC GAAATATCTT [reverse]; amplifying 125 bp); (ii) the *I. ricinus* nuclear gene *cal* (primers ATCTCCAATTTCGGTC CGGT and TGAAAGTTCCCTGCTCGCTT; amplifying 109 bp); and (iii) the *I. ricinus* mitochondrial gene *COII* (primers CCGACTTCTTGACGTAGACAAC and CTGATTAAGGC GACCAGGAACG; amplifying 144 bp). PCR cycling for *gyrB* and *cal* was as follows: 95°C for 2 min, 40 cycles at 95°C for 15 s and at 60°C for 30 s, and melt curve from 55°C to 95°C with increasing increments of 0.5°C per cycle. The cycling for *COII* differed only in the annealing temperature, set at 58°C. All reactions were performed in 25 μl of Milli-Q water containing 400 nM of each primer, 12.5 μl of iQ Sybr green supermix, and 1 μl of DNA. PCR products were sequenced to confirm PCR specificity and then ligated into the pGEM-T Easy vector and cloned. Purified plasmids containing the desired fragments were serially diluted to evaluate the efficiency and detection limit of each PCR protocol (10 copies in each case). PCRs were then performed on each tick DNA sample in triplicate. PCR efficiency was assessed by serial dilution of samples from each life stage subset. Using the software SPSS version 14.0, the nonparametric Kruskal-Wallis H test and the Mann-Whitney U test were used to compare genome copy numbers for each life stage; P values of ≤ 0.05 were considered to be significant. A total of 156 *I. ricinus* samples from 12 different life stages were examined. Threshold cycle values were found to be highly reproducible for all three protocols, with mean intra- and interassay coefficients of variation always less than 2% and 5%, respectively.

Estimates of the total number of symbiont, nuclear, and mitochondrial genome copies were obtained via a comparison of the qPCR results of each tick life stage with those of serial dilutions of cloned fragments (containing known copy numbers). Although we did not determine how many genome copies each "*Ca.* Midichloria mitochondrii" cell contains, other members of the *Rickettsiales* are known to have a single genome per cell (10) and a single copy of the *gyrB* gene (2). Thus, the *gyrB* copy number can be assumed to be approximately equivalent to bacterial numbers or at least directly correlated with them. Since mitochondria can contain a variable number of genome copies (3a), we can consider the *COII* gene copy number as an approximation of the number of mitochondria present in tick samples.

Life stages and qPCR results for each gene are shown in Table 1. The copy numbers of both *cal* and *COII* rise during the development of the tick from egg to adult, with notable increases following each molt and constant numbers during each stage. In adult females, 5 days after detachment, there

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FIG. 1. The *gyrB*/*cal* (A) and *COII*/*cal* (B) ratios in the various *I. ricinus* life stages, determined by qPCR. Abbreviations: Ed1, eggs day 1; Ed15, eggs day 15; Ld1, larvae day 1; Leng, engorged larvae; Nd1, nymphs day 1; Neng, engorged nymphs; Fne, nonengorged females; Fpe, partially engorged females; Fd5ad, engorged females 5 days after detachment; Fd10ad, females 10 days after detachment; Fadep, females after egg deposition; M, males positive for "*Candidatus* Midichloria mitochondrii." The boxes represent the 25th and 75th percentiles of the values, with the line inside the boxes marking the median. The whiskers indicate the 10th and 90th percentiles. Outlying points are represented by ● or ⋆.

TABLE 1. Median values and ranges of *gyrB*, *cal*, and *COII* copy numbers and of *gyrB*/*cal* and *COII*/*cal* ratios[a]

Stage (no. of replicates)	*gyrB* copy no.		*cal* copy no.		*COII* copy no.		*gyrB*/*cal* ratio		*COII*/*cal* ratio	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Eggs, day 1 (17)	2.0×10^6	7.0×10^5–4.2×10^6	5.4×10^2	9.3×10^1–7.0×10^3	6.4×10^6	1.2×10^5–2.4×10^7	3.4×10^3	1.4×10^2–2.2×10^4	8.3×10^3	5.1×10^2–1.3×10^5
Eggs, day 15 (15)	2.9×10^6	5.4×10^5–6.7×10^6	3.4×10^3*	1.8×10^3–1.3×10^4	2.8×10^6*	1.3×10^5–6.0×10^6	9.2×10^2*	4.3×10–$3,537.3$	8.0×10^2*	6.0–32.1×10^3
Larvae, day 1 (13)	2.5×10^4*	7.6×10^3–6.6×10^4	1.3×10^5*	1.9×10^4–1.5×10^5	1.9×10^7*	5.6×10^6–2.9×10^7	0.2*	0.1–3.5	2.4×10^2	6.2×10–7.3×10^2
Larvae, engorged (18)	1.8×10^5*	7.8×10^4–4.3×10^5	1.3×10^5	2.4×10^4–4.0×10^5	4.2×10^7*	2.5×10^7–4.5×10^7	1.6*	0.4–6.8	2.6×10^2	1.1×10^2–3.4×10^2
N, day 1 (18)	1.9×10^4*	3.2×10^3–1.6×10^5	5.0×10^5*	3.4×10^5–1.1×10^6	8.2×10^7	96.9×10^3–24.5×10^7	0.03*	0.0–0.4	1.3×10^2	0.2–7.1×10^2
N, engorged (18)	2.9×10^5*	1.3×10^4–1.0×10^6	5.0×10^5	1.7×10^5–9.3×10^5	6.8×10^8*	3.3×10^8–1.2×10^9	0.7*	0.0–1.6	1.4×10^3*	9.2×10^2–1.7×10^3
F, unfed (13)	2.3×10^6*	7.2×10^5–8.3×10^6	3.4×10^6*	2.1×10^6–6.1×10^6	1.1×10^9	2.2×10^7–2.2×10^9	0.8	0.2–2.0	2.4×10^3*	7.4–5.3×10^2
F, partially engorged (13)	7.0×10^6	5.2×10^5–2.5×10^7	2.0×10^6*	4.6×10^5–4.0×10^6	2.0×10^9	9.6×10^7–5.2×10^9	3.2*	0.9–20.1	6.3×10^2*	2.1×10^2–4.3×10^3
F, 5 days after detachment (11)	3.3×10^6	1.7×10^6–2.4×10^7	2.0×10^5*	9.3×10^4–8.9×10^5	10.0×10^7*	7.7×10^6–2.5×10^8	2.0 × 10*	5.7–28.9	2.5×10^2	0.9–7.6×10^2
F, 10 days after detachment (11)	1.4×10^7*	1.0×10^6–1.1×10^8	2.9×10^6*	3.1×10^5–1.1×10^7	9.4×10^8*	2.2×10^8–2.2×10^9	4.7*	1.6–10.9	1.8×10^2	7.8×10–3.0×10^2
F, after deposition (4)	7.9×10^7*	3.5×10^7–1.5×10^8	3.1×10^6	7.0×10^5–1.1×10^7	1.6×10^9	3.1×10^8–5.6×10^9	2.5×10*	1.4×10–50.1	4.8×10^2*	4.1×10^2–6.0×10^2
Adult males (15)	3.2×10^3*	0–4.3×10^5	1.3×10^6	4.9×10^5–2.1×10^6	2.1×10^8*	6.5×10^6–3.2×10^8	0.003*	0.0–0.0	1.3×10^2	0.1–6.6×10^2

[a] N, nymphs; F, females; *, statistically significant difference with respect to the previous stage.

is a drop in the copy number of *cal*. This is likely a result of extensive apoptosis in the salivary glands and other tissues (6, 7). Adult females have significantly higher *COII* copy numbers than males, presumably due to the energy requirements of oogenesis. The *gyrB* copy number is relatively high in eggs and drops markedly in newly hatched larvae. High concentration in the eggs is typical for vertically inherited symbionts (4) and may reflect competition among symbionts for transmission to progeny (14). The drop in the copy number of *gyrB* from the egg stage to the larval stage is presumably due to bacteria being excluded from most tissues of the embryo during development. The *gyrB* copy number then increases following engorgement of larvae. Following molting to the nymph stage, the *gyrB* copy number drops again but increases again following engorgement of nymphs. Following the molt to the adult female, the *gyrB* copy number increases. This is probably due to the fact that the ovaries, the primary niche of the symbiont, are fully

developed in adults. The *gyrB* copy number continues to increase in females during engorgement and egg deposition.

We were not able to determine the sex of larvae and nymphs. The available evidence indicates that the symbiont does not cause sex ratio distortion (i.e., via male-killing, parthenogenesis, or feminization) (8). Based on the relatively low variance of the *gyrB* copy number in larvae, it appears that the symbiont is transferred to both male and female larvae. However, the higher variance in nymphs, combined with the large difference in the *gyrB* copy numbers between adult females and males, suggests a specialization toward females during the nymph stage. Nine of 15 adult males were found positive for the symbiont, but these had the lowest *gyrB* copy numbers of all life stages examined.

The *gyrB*/*cal* and *COII*/*cal* ratios are shown in Fig. 1. The *gyrB*/*cal* ratio was highest in day-old eggs and follows a similar pattern to the *gyrB* copy number (Table 1). One exception is the large jump in females 5 days postdetachment, which is due to the large drop in the *cal* copy number at this stage (see above). The *COII*/*cal* ratio is highest (10^4) in day-old eggs and then drops to a stable level of 10^2 to 10^3 in other stages. The fact that both the *gyrB*/*cal* and *COII*/*cal* ratios are highest in day-old eggs is interesting, given the tendency of "*Ca.* Midichloria mitochondrii" to invade mitochondria. The behavior of the symbiont in eggs has not yet been examined.

In conclusion, the increase in the *gyrB* copy numbers following engorgement of each of the three stages indicates that bacterial growth is linked to the blood meal. Whether this coincides with the production of metabolites by "*Ca.* Midichloria mitochondrii" for its host remains to be determined. The growth may also reflect competition among symbionts for transmission to the next stage of the tick, although the increase appears to occur in both female and male larvae.

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